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The DNA replication factor RPA physically associates with the tumor suppressor protein p53, an interaction that could be important for the function of both these proteins in normal and cancer cells. We have created two mutant forms of p53 with the desired transcriptional activation property. Thus we are now in the position to use these mutant versions of p53 to test whether the RPA binding ability of p53 is important for its multiple functions. Deletion fragments of Rpa1 were made by in vitro transcription translation to determine which parts of Rpa1 bound to p53. Binding to p53 requires a region of Rpa1 overlapping with the high affinity binding site for DNA explaining how p53 could competitively interfere with the DNA binding function of RPA. The evolutionarily conserved putative zinc finger near the C terminus of Rpa1 was not required for binding to p53. We have also extended our study to the p21 protein, which is induced by p53 and interacts with both the cdk2 kinase, and a DNA replication factor, PCNA. Domains of the p21 protein have been defined that execute these functions independently.

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FOREWORD

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J.C Aug-2-95

PI - Signature Date

TABLE OF CONTENTS

The following work was done under the supervision of Dr. Anindya Dutta, who is currently supported by a Career Development grant (DAMD17-94-J-4064). I am a postdoctoral fellow in Dr. Anindya Dutta's lab. Our independent grants only support our own salaries. The first part of the work on the interaction of tumor suppressor p53 and Replication Protein A (RPA) was done in concert with Dr. Anindya Dutta, based on the earlier finding by Dr. Dutta that p53 can interact with RPA and inhibit its function. In the following report, I have summarized results from this study and referenced Dr. Dutta's annual report for detailed information. Our interests in the function of p53 in the development of breast cancer led us to investigate an important cell cycle regulatory protein p21, which is believed to be the major downstream regulatory protein of p53 in the control of cell cycle. This part of the work is unique to my grant.

INTRODUCTION

Tumor Suppressor p53 has been the major focus of the cancer research, because it is mutated in more than 50% of human cancers including breast cancer (1). There is significant evidence suggesting the importance of p53 in the cancer development: deletion of p53 gene in "knock-out" mice confers a very high risk of cancer; several viral oncogenes, including E6 gene of human papilloma virus and large T antigen of Simian virus 40, can specifically inactivate p53 by various mechanisms; cellular oncogene mdm2 can specifically bind to the N-terminal part of p53 and inactivate its transactivation activity. p53 has multiple biological functions: overexpression of p53 can arrest cell cycle at G1 to S transition; p53 is essential for DNA repair after mild DNA damage; p53 is also required for apotosis following extensive DNA damage. The transforming mutants of p53 are defective in all these functions.

The mechanisms by which p53 carry out these biological functions are still unclear. p53 is a sequence-specific DNA binding protein. p53 is transcriptional activator, that has been shown to activate a number of cellular genes including cellular oncogene mdm2, DNA repair gene GADD45 and cell cycle regulatory p21 gene. p53 can also bind to TATA-box binding protein TBP, and suppress general transcription from promoters lacking p53 binding sites. Both transcription activation and suppression activity of p53 can contribute to its various biological functions. Two years ago, our group (2) found that p53 could directly bind to DNA Replication Protein A (RPA) and inhibit its activity. These findings suggested a novel mechanism of p53 function, that is inhibiting DNA replication by directly interacting with RPA.

Replication Protein A (RPA, also called RF-A or human ssb) is a complex of three polypeptides of 70 kD, 34 kD and 13 kD, essential for SV40 DNA replication *in vitro* and excision repair in animal cells. In *S. cerevisiae* the genes for the 70, 34 and 13 kD subunits are each essential for viability (3). RPA functions as a single strand DNA binding protein. p53 can binds to RPA and inhibit its ability to bind to single-stranded DNA. Most of the transforming mutants of p53 fail to inhibit DNA replication *in vivo* and *in vitro* and fail to bind to the specific p53 binding DNA element. However, the two transforming mutants of p53 (R175H and R273H) we tested could still interact with and inhibit RPA *in vitro*. Therefore the ability to interact with RPA is not the sole mechanism by which p53 inhibits cellular DNA replication, because mutant p53 which can still bind RPA fails to inhibit replication. We hypothesize that p53 has to be capable of binding RPA *and* DNA to simultaneously in order to inhibit the DNA replication apparatus. The transforming mutants of p53 do not bind DNA and so fail to be near origins of replication where the inhibition of RPA has the most profound effect on DNA replication. In this hypothesis, the ability to interact with RPA is still important for the replication inhibitory property of p53, and the prediction is that point mutant forms of p53 which can still bind to DNA, but fail to bind RPA, will have lost the growth suppression function. This hypothesis is tested here.

The transcriptional activity of p53 results in the increased expression of several human genes, one of which is the cell-cycle regulator, p21. Transcription of p21 is induced by p53 upon DNA damage (4). "knock-out" experiment show that p21 is required for cell cycle arrest upon DNA damage (5, 6). Overexpression of p21 can arrest cells at G1-S transition just like p53 (4, 7, 8). All these data suggest that p21 protein is a major effector of the growth suppression function

of p53. p21 interacts with and inhibits the cyclin dependent kinases (cdk), and also interacts with and inhibits a DNA replication factor PCNA (9-12). Because of the possibility that this pathway from p53 to the DNA replication apparatus may be as important as the direct interaction between p53 and RPA, we have begun investigating whether the p21-PCNA interaction contributes to growth suppression.

BODY

SPECIFIC AIMS FOR YEAR 1

1. Define the part of p53 involved in binding RPA and mutate it to obtain mutant forms of p53 which do not bind RPA. (Year 1, Task 1)
2. Analyze the effect of these mutations on the transcriptional property of p53. (Year 1, Task 1)
3. Define the part of Rpa1 that binds p53. (Year 2 Task 1)
4. Examine whether the p21-PCNA interaction contributes to growth suppression by p21.

RESULTS

Studies on the interaction of p53 and RPA:

(The specific aims 1 to 3 for year 1 are the work on the interaction between tumor suppressor p53 and Replication Protein A (RPA). These studies were done in concert with Dr. Anindya Dutta, who is my postdoctoral advisor. I summarized our results in the following paragraph and referenced Dr. Dutta's annual report DAMD17-94-J-4064 for detail information.)

Earlier study from our lab showed that tumor suppressor p53 could interact with Replication Protein A (RPA) and inhibit its function as a single strand DNA binding protein. We propose that p53 inhibits entry into S phase by its interaction with RPA. To test this hypothesis, we defined the domain of p53 that binds RPA. Deletion mutagenesis showed that N-terminal domain (amino acids 2-71) of p53 was necessary and sufficient for binding of p53 to RPA in crude cell extract. Point-mutations in amino acids 53-54 (W53S-F54S) disrupted the interaction. Mutations in amino acids 48-49 (D48H-D49H) also decreased RPA binding. The mutations which changed amino acids 22-23 of p53 (L22Q-W23S) affect its ability to activate transcription, but did not affect its ability to bind RPA. Transient transfection assay was done to test the transcription activation properties of these p53 mutants. Only the L22Q-W23S mutation of p53 significantly affected transcription activation by p53 (Dr. A. Levine's lab). D48H-D49H and W53S-F54S mutant forms of p53 retained 50-100% of transcriptional activity compared to wild-type p53. Therefore we separated the transactivation and RPA binding functions of p53 with appropriate point mutations. Now, we had mutations of p53 with near wild-type transactivation but diminished RPA binding activities.

p53 binds RPA through direct interaction with RPA1, the largest subunit of RPA. RPA1 can bind to single-stranded DNA on its own. One model that p53 can inhibit the single-stranded DNA binding ability of RPA is by competing for the same region required for the single-stranded DNA binding. To test this, deletion mutagenesis was used to define domains required for p53 binding, ssDNA binding and complex formation (RPA1 from a complex with two other polypeptides, RPA2 and RPA3). The results suggest that the p53 binding domain significantly overlaps with the domains required for ssDNA binding, but not with the domain required for complex formation. This agrees with our earlier results that p53 can bind to RPA holocomplex, and inhibit its ssDNA binding activity.

Separate domains of p21 involved in the inhibition of cdk kinase and PCNA.

p21 (WAF1, CIP1 or sdi1) is a protein induced by the tumor suppressor protein p53, which interacts with and inhibits two different targets essential for cell-cycle progression . One of these is the cyclin-cdk family of kinases and the other is the essential DNA replication factor PCNA. To examine the contribution of each these two activities to the growth suppression function of p21, we separated domains of p21 responsible for interacting with and inhibiting these two targets and tested their function *in vivo*.

The N terminal half of p21 binds to and inhibits cdk2 kinase.

When the N or C terminal halves of p21 (p21N or p21C) were expressed separately as GST fusion proteins, p21N alone physically associated with bacterial expressed cdk2 kinase polypeptide in a standard "pull-down" assay (Figure 1). Further, p21 and p21N, but not p21C, inhibited the kinase activity of cyclin A-cdk2 (Figure 1) or cyclin E-cdk2 (data not shown). Thus the N terminal domain (amino acids 1-90) inhibits cyclin-cdk kinases.

The C terminal half of p21 binds to PCNA and inhibits SV40 based DNA replication reaction.

The ability to interact with PCNA is the function of the C terminal half of p21 (p21C). In a pull-down assay bacterial expressed PCNA was quantitatively bound by p21 and p21C (Figure 2). As reported, GST-p21 inhibited the SV40 based *in vitro* DNA replication reaction , and this inhibition was reversed by the addition of PCNA or a fraction of cell extract containing PCNA (data not shown). The ability to inhibit the SV40 based DNA replication reaction required the PCNA binding domain present in p21C (Figure 2). Although the concentration of p21N added was sufficient to completely inhibit the endogenous histone H1 kinase activity in the extracts (data not shown), SV40 DNA replication was not inhibited. Thus the C terminal domain of p21 (amino acids 87-164) binds to and inhibits PCNA.

Inhibition of Xenopus DNA replication by different domains of p21.

p21 inhibits DNA replication of sperm in interphase extracts derived from *Xenopus* eggs (10). p21N inhibits DNA replication in the *Xenopus* extract at the same concentrations as p21 (Fig. 3a), which are similar to those of cyclin E-cdk2 (100 nM). Thus, in *Xenopus* extracts it appears that cyclin-cdk2 rather than PCNA is limiting and is inhibited by p21. High concentrations of p21C, approximating that of PCNA in the extract (10 μM), inhibited *Xenopus* DNA replication. Therefore, PCNA is also required for double-stranded DNA replication and can be inhibited by p21, but is not the limiting factor inhibited by the addition of p21. In contrast to double-stranded DNA replication, DNA synthesis on single-stranded DNA was not inhibited by the cdk inhibitory p21N domain. but was inhibited by p21C at concentrations approaching that of PCNA (Fig. 3b). Therefore active cdk kinase is required specifically for DNA synthesis on double-stranded DNA, while PCNA is required for DNA synthesis on both types of substrates.

Growth suppression assay show that p21N inhibits cell growth while p21C does not.

Using these separated domains, we have determined that p21 inhibits different biological systems through different targets. The question arises as to which of the two targets, cdks or PCNA, mediates the growth suppression function of p21. We tested this in p53 null transformed cells in culture (Fig. 4a). As reported by others (4, 7), plasmids expressing p21 established fewer colonies compared to control vector plasmids. The N terminal cyclin-cdk2 inhibitory domain (p21N) also suppressed growth, and the C terminal PCNA inhibitory domain (p21C) did not, suggesting that cyclin-cdks are the primary targets of p21 in transformed cells. An immunoblot of extracts of transfected COS cells shows that human p21, p21N and p21C are expressed from these plasmids and that the exogenous human p21 and p21C proteins associated with and co-immunoprecipitated cellular PCNA (Fig. 4b). Therefore, the failure to suppress growth by p21C is not due to low level of expression or denaturation of truncated protein p21C *in vivo*. p21C reproducibly stimulated the number of colonies obtained upon transfection, but the significance of this is unclear.

Further mapping of PCNA-binding domain in p21 suggests that C-terminal 38 amino acids are necessary and sufficient.

Although inhibition of PCNA by p21C is not apparently required in the particular growth suppression assay used above, PCNA-binding activity of p21 may be required in some aspects of the cell cycle, such as transition from quiescence stage to cycling stage. In order to study the PCNA-p21 interaction in more detail, we decided to further map the PCNA-binding domain in p21. Bacterially expressed glutathione-S-transferase-p21 (GST-p21), GST-p21C and GST-p21C2 were used as an affinity matrix to demonstrate that PCNA interacts with the last 39 amino acids of p21 (Fig. 5 and data now shown). Scatchard analysis of the interaction (at 4^{o}C) showed that the Kd of p21C-PCNA and p21C2-PCNA interactions were 15.4 nM and 12.0 nM respectively (Fig. 6). A synthetic 41 amino acid peptide corresponding to p21C2 (plus two lysines at the C terminus) was synthesized. This synthetic peptide competitively inhibited the binding of PCNA to GST-p21 at 4^{o}C (Fig. 6).

These experiments demonstrate that the C terminal 39 amino acids of p21 are necessary and sufficient to bind PCNA.

FIGURE LEGENDS

Fig. 1 The N terminal half of p21 binds to and inhibits cdk2 kinase. **a** Immunoblot probed with anti-cdk2 antibody shows which GST fusion proteins bind cdk2. Ponceau S stain of the same blot demonstrates the relative amounts of the various GST fusion proteins present in each lane. The GST lane also contains molecular weight markers, and the 45 kD marker is indicated. The intense band in the 0.1 input lane is the carrier protein casein. **b** Autoradiogram of proteins showing the phosphorylation of histone H1 by cyclin A-cdk2 kinase with the following additions. Lane 1, no addition; 2, 3, 20 ng and 100 ng GST-p21; 4, 100 ng GST; 5-7, 20 ng, 100 ng, 200 ng GST-p21N; 8-10, 20 ng, 100 ng, 200 ng GST-p21C.

Fig. 2 The C terminal half of p21 binds to PCNA and inhibits SV40 based DNA replication reaction. **a** Immunoblot probed with anti-PCNA antibody (Oncogene Science) shows which GST fusion proteins bind PCNA. Ponceau S stain of the same blot demonstrates the relative amounts of the various GST fusion proteins present in each lane. **b** Titration of various fusion proteins into SV40 DNA replication reactions containing SV40 T-antigen and a crude cell extract from human 293 cells. DNA synthesis is expressed as picomoles of dAMP incorporated in a 50 µl reaction in 1 hour.

Fig. 3 **a** p21N inhibits double stranded sperm DNA replication in *Xenopus* egg extracts at similar concentrations as full length p21. p21C inhibits DNA replication only when added at concentrations approaching that of PCNA in the *Xenopus* extracts. **b** p21C but not p21N inhibits DNA synthesis on single-stranded DNA at concentrations similar to that required to inhibit DNA replication on double-stranded DNA.

Fig. 4 **a** p21N inhibits cell growth while p21C does not. Number of G418 resistant colonies obtained after transfection of SaOs2 cells with equal amounts of indicated plasmids is shown relative to the number obtained with vector control alone (cDNA3). Mean and standard deviation of five independent transfections. **b** Upper: Immunoblot probed with anti-p21 polyclonal antibody shows p21 and its derivatives were expressed *in vivo*. The 21 kD polypeptide seen in all lanes could be monkey p21. Exogenous proteins of 21, 13 and 13 kD are also seen in cells transfected with p21, p21N and p21C, respectively. Lower: The C-terminal half of p21 formed a complex with PCNA *in vivo*. Transiently transfected cell lysates were immunoprecipitated with anti-p21 polyclonal antibody and the precipitate immunoblotted with anti-PCNA antibody. All lanes contain PCNA presumably co-immunoprecipitated with monkey p21. Cells expressing p21 and p21C co-immunoprecipitate larger quantities of PCNA due to association of PCNA with the exogenous protein.

Fig. 5 Deletion analysis of p21 shows that the C terminal 39 amino acids is necessary and sufficient for binding PCNA. **a** Immunoblot with anti-PCNA antibody. The indicated GST fusion proteins were used to mediate the binding of bacterially produced human PCNA (37 kD) to glutathione agarose beads. One-tenth of input PCNA is shown for comparison. The smaller band seen in the second lane is the GST-p21N protein which is about 35 kD in size. Due to their high protein content, GST fusion proteins produce background bands in the Enhanced Chemiluminescence reaction used to visualize the immunoblots. **b** Schematic summary of deletion derivatives of p21 and their ability to bind PCNA (Figure 1A and data not shown). The numbers indicate which amino acids of p21 are present in the deletion derivatives.

Fig. 6 **a** Scatchard analysis of the binding of PCNA to GST-p21C or GST-p21C2 at 4°C. The Y-axis shows the ratio of bound PCNA (cpm/200 µl) to the total concentration of the GST-fusion protein (nM) (b/R_t). The X-axis shows the amount of bound PCNA (cpm/200 µl) (b). Each point is the mean and standard deviation of 4 measurements, and the slope of the line equals -

1/Kd. **b** Synthetic p21C2 peptide can competitively inhibit p21-PCNA interaction at 4°C. Binding of PCNA visualized by immunoblotting of bead-bound proteins with anti-PCNA antibody. 1 μM GST-p21 was incubated at 4° C with 100 μg of S100 extract from 293 cells. Concentrations (μM) of peptide competitor are indicated at the top: p21C2 peptide (lanes 2, 3) or negative control peptide CSH262 (lanes 4-7). Lane 1, one-tenth input lysate; lane 2, bound to GST protein; lanes 3-9, bound to 1 μM GST-p21 protein. Competing peptides were none (lanes 1-3) and indicated concentrations of p21C2 or negative control peptide CSH262.

Figure 1

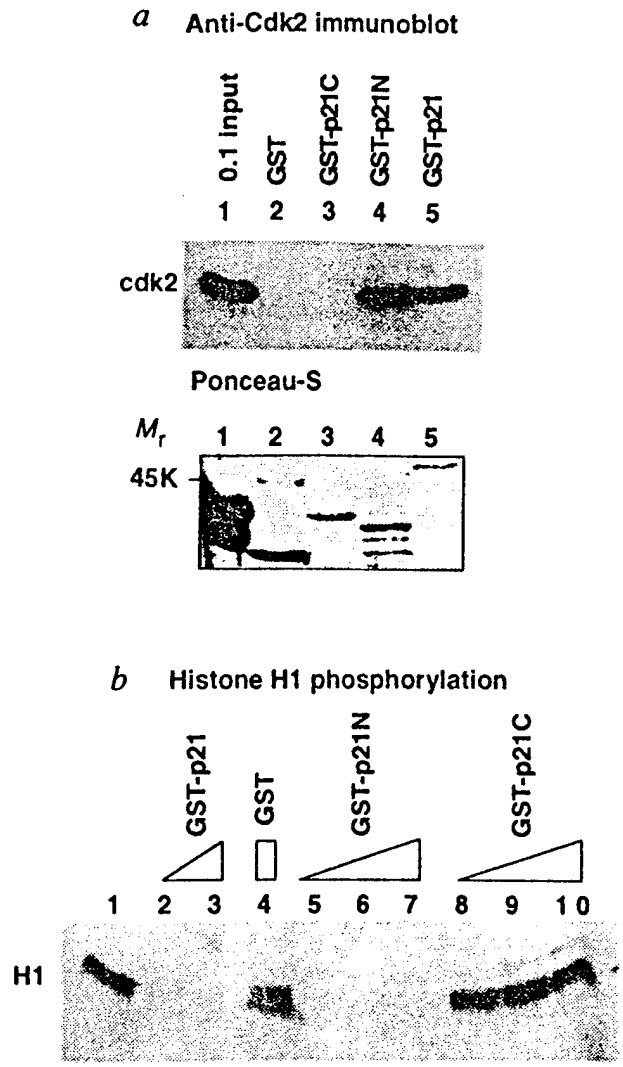
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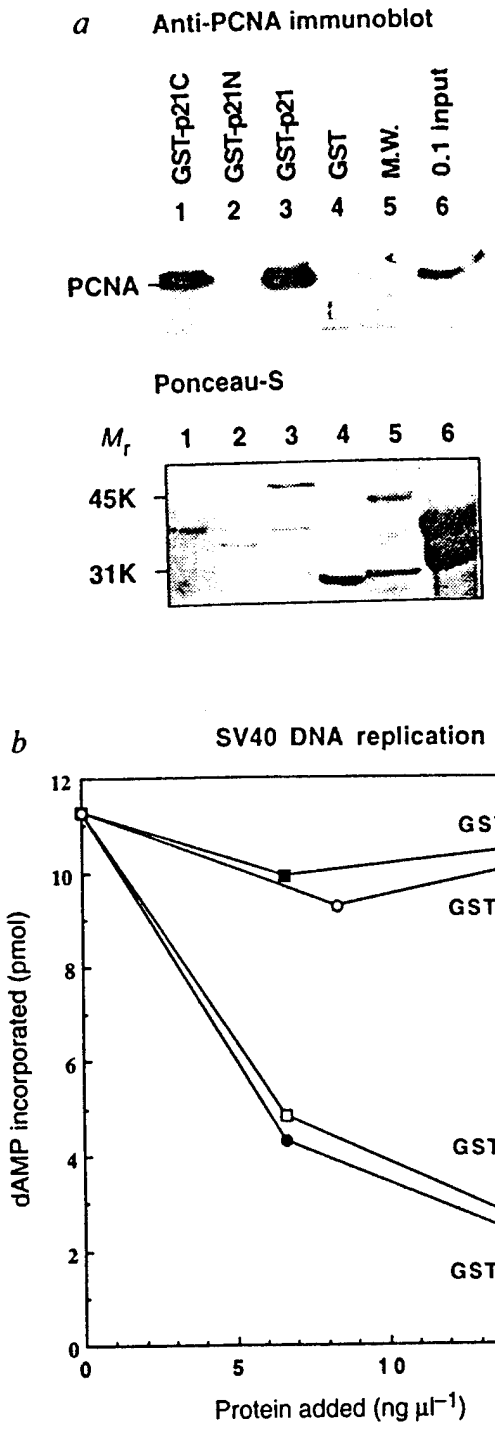
Figure 2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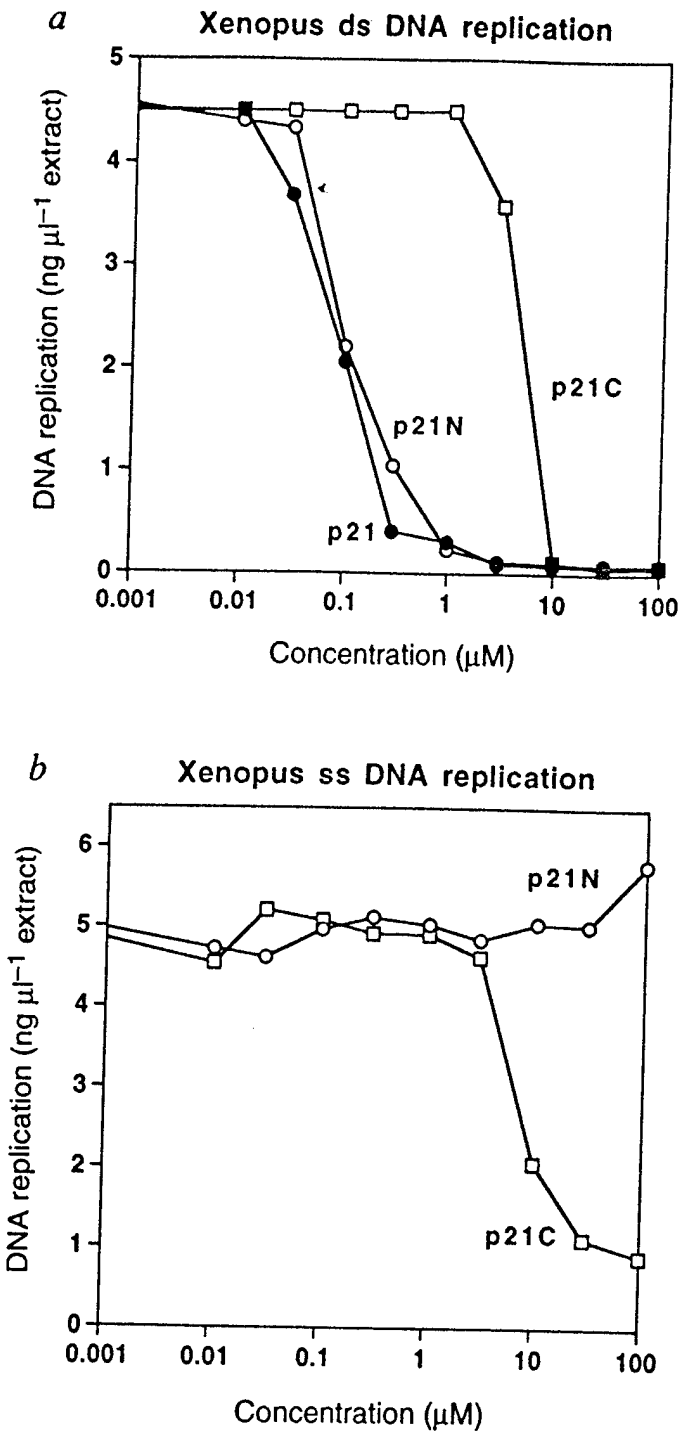
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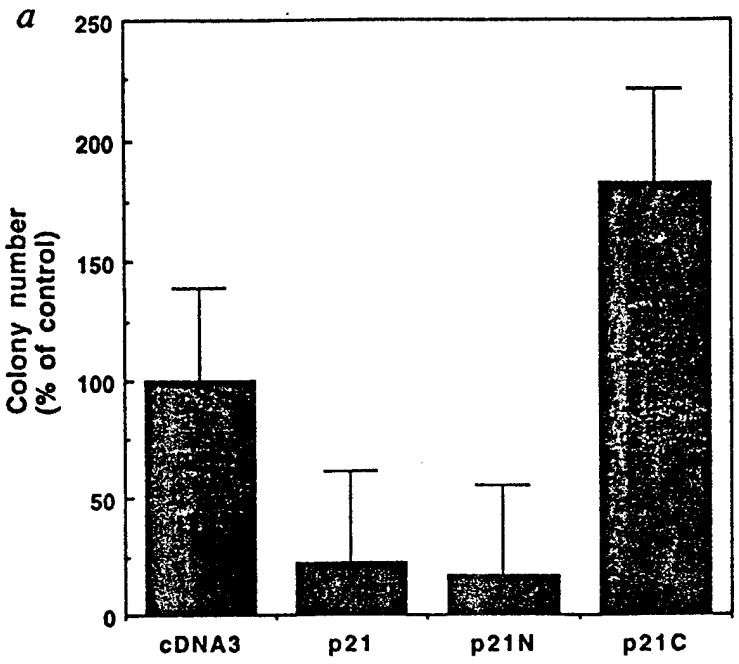
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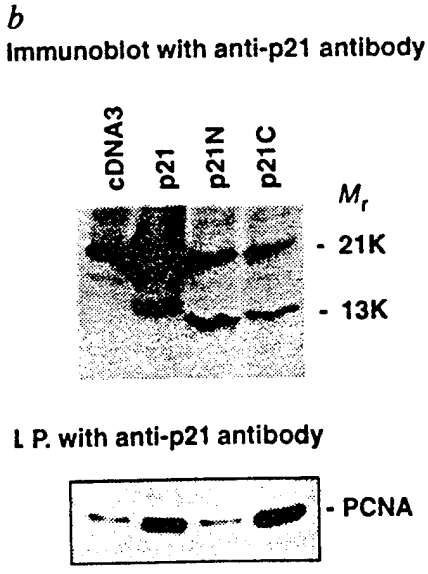
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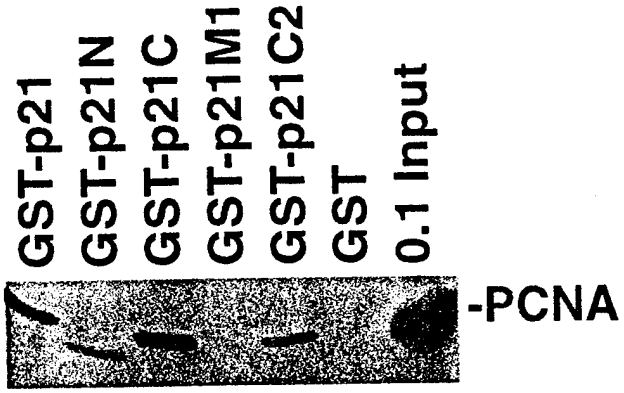
Figure 5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GST-p21C2
GST
0.1 Input
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p21N (1-90) -
p21N1 (1-129) -
p21N2 (1-150) -
p21C (87-164) +
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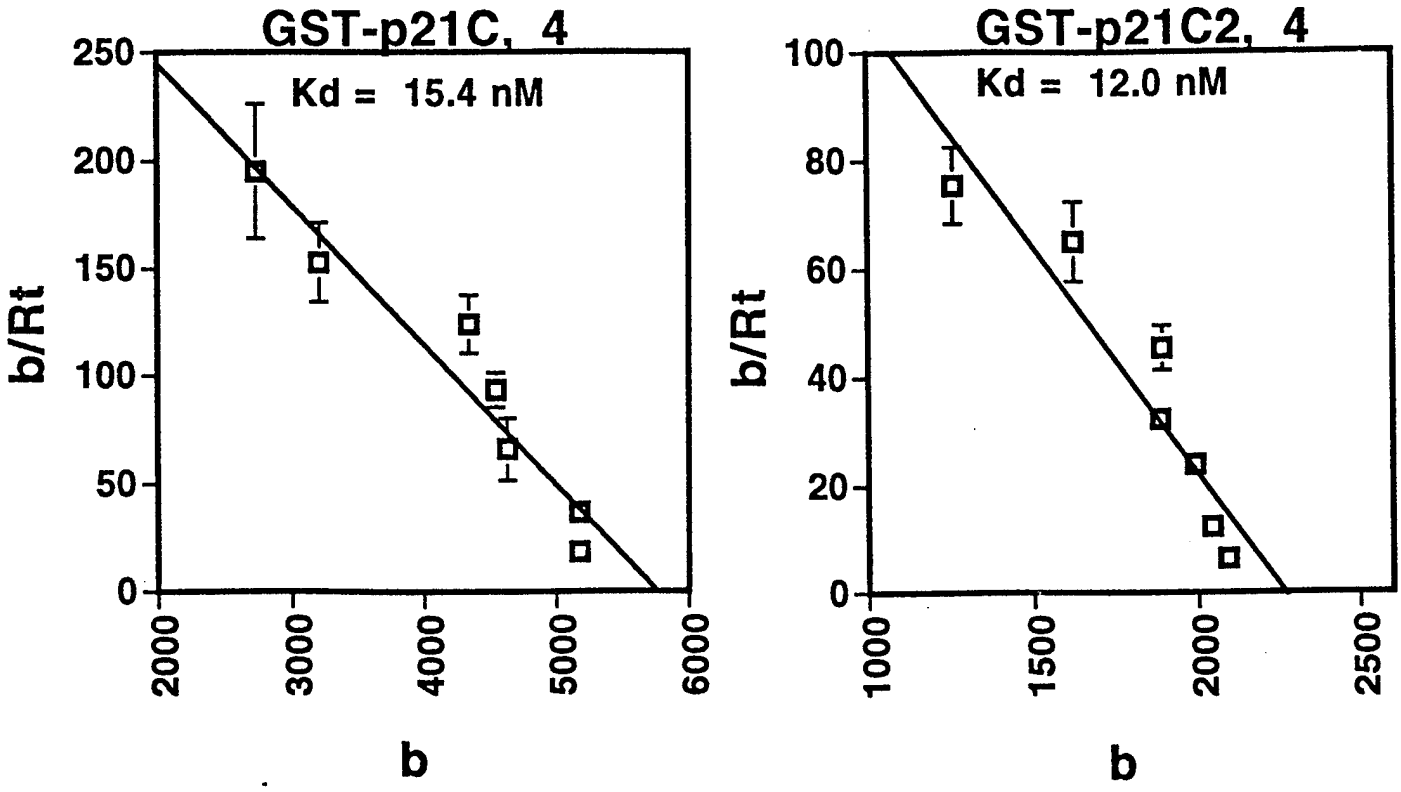
p21C2 (126-164) +
p21M (55-150) -
p21M1 (55-129) -

Figure 6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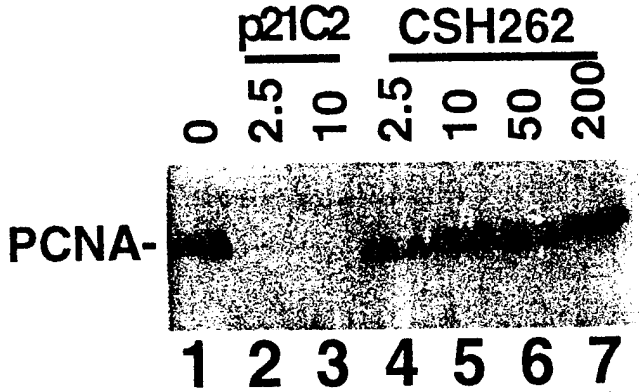
Kd = 15.4 nM
b/Rt
b
GST-p21C2, 4
Kd = 12.0 nM
b/Rt
b


p21C2
CSH262
0
2.5
10
2.5
10
50
200
PCNA-
1 2 3 4 5 6 7

CONCLUSIONS

In the first year of our study, we defined the domain of p53 that bound to RPA. Mutation of aromatic residues or surrounding negatively charged residues in this domain abolished the interaction. These point mutations still retain most of the transactivation activity of p53. One mutation generated by Dr. A. Levine' lab, L22Q-W23S showed decreased transcriptional activation but wild-type RPA binding indicating that transcriptional activation function is not required for RPA binding. Therefore we separated the transactivation activity and RPA binding activity of p53. In the next two years, we are going to determine how these mutations of p53 affect other functions of p53, including transcription suppression, cell cycle arresting, and induction of apoptosis upon extensive DNA damage.

We also defined domains in RPA1 that required for RPA complex formation, ssDNA binding and p53 binding. The separation of these functional domains make it possible to screen for point mutations in RPA1 that retain all the other functions except p53 binding. These mutations can be used to examine the importance of p53-RPA interaction on RPA function in DNA replication, repair and recombination.

p21 has been shown to be the major downstream regulatory factor of p53. Having shown that the inhibition of cyclin-cdk and inhibition of PCNA are independently executed by two different domains of p21, we could determine which target mediates the effect of p21 in each biological system. cdk kinases are the primary target for inhibition of double-stranded DNA replication in *Xenopus* extracts by p21 and for growth suppression in transformed cells. In contrast, PCNA is the limiting target in the SV40 replication reaction. Inhibition of PCNA by p21 (and p21C) is not apparently required in the particular growth suppression assay used in this report. Transformed cells may have an excess of PCNA, so that higher levels of p21C are required to inhibit PCNA function. Inhibition of PCNA may also be more important for the p21 mediated temporary switch from DNA replication to repair following genotoxic damage. Further mapping of PCNA-binding domain suggests that C-terminal 38 amino acids are necessary and sufficient for the binding of PCNA. More sensitive assay, such as microinjection, will be used in the future to test whether PCNA binding activity of p21 can inhibit DNA replication *in vivo*.

The ability of p21N alone to inhibit cell-cycle progression has interesting implications for the activity of other cdk-inhibitory and cell-cycle blocking polypeptides like p27 (up-regulated by TGFb, (13, 14)) and p57 (15, 16) which show homology with p21 in the region contained in p21N. Currently, we are narrowing down the cdk2 inhibitive domain to design small peptides that can inhibit the cdk kinase activities. Such peptides can lead to the development of therapeutic chemicals that targeting the cell cycle machinery.

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Appendix (annual report for DAMD17-94-J-4064)

This work is being done in concert with a postdoctoral fellow in my laboratory, Dr. Junjie Chen, who is independently supported by a postdoctoral fellowship: DAMD17-94-J-4070. He is revising his report to ensure that it is significantly different from this one. However, there is significant overlap in the work described because it is being done in concert by a postdoctoral fellow (Dr. Junjie Chen) and a principal investigator (Dr. Anindya Dutta). The grants support our separate salaries.

INTRODUCTION

Importance of p53 function in breast cancer: The p53 protein is an important tumor suppressor which is inactivated by mutation in up to 50% of breast cancers [1-4]. The mutated p53 is often over-expressed, so that intense staining with anti p53 antibodies upon immunohistochemistry of breast cancers has been used as a marker for such inactivation. When this issue was examined directly, 100% correlation was found in 15 patients between p53 accumulation and p53 mutation [5] Cytogenetic, and DNA analysis techniques have detected loss of heterozygosity of the p53 locus (17p13.1) in up to 64% of primary breast cancers [6]. Such a loss of heterozygosity is also considered a marker for inactivation of a tumor suppressor gene, because the remaining allele of the gene usually harbors an inactivating point mutation. Direct analysis of the p53 gene by PCR amplification of tumor DNA has shown mutations in 32% of invasive and 12% of intraductal/predominantly intraductal breast carcinomas, implying that mutation of the p53 gene is important for breast cancer progression [7]. Over-expression of p53 in breast cancer has been associated with increased proliferation: the % of cells in S phase being 7.1% in p53 positive tumors vs 4.1% in p53 negative tumors [3]. In multiple studies, mutation of the p53 gene in a breast cancer is associated with shorter disease free interval and decreased overall survival, independent of the presence of axillary node metastases [3, 4, 10, 11]. In one study, for example, immunohistochemistry on paraffin sections in 289 axillary node negative breast cancer cases showed increased levels of p53 in 41 patients. p53 positive tumors were associated with an eight year survival of 56% as opposed to 81% for p53 negative tumors [11].

In a small group of familial breast cancer patients (with Li Fraumeni syndrome), inactivating germ-line mutations are seen in the p53 gene, indicating the importance of normal p53 in preventing the appearance and progression of breast cancers [12, 13]. Cells from patients with Li-Fraumeni syndrome are heterozygous (mutant/wild type) for p53 in somatic non-neoplastic tissue, and homozygous for mutant p53 in tumor tissues. However, germ-line mutation of p53 is not a common cause of familial breast cancers [14]. Finally, the introduction of wild type p53 into breast cancer cell lines with mutant p53 reversed their ability to form colonies in soft agar and produce tumors in nude mice [15]. This indicates that restoring normal p53 (or some of the functions carried out by p53) to breast cancers could have therapeutic value.

Finally, in a study of 27 cases of inflammatory breast cancer showed that 30% had increased p53 in the nucleus (mutant p53), and 33% had no p53 staining [16]. The surprising result was that 37% of the tumors had wild type p53 which showed cytoplasmic staining with nuclear sparing. Therefore, beside the mutational inactivation of p53, the tumor suppressor may also be inactivated in breast cancers by post-translational processes which interfere with nuclear localization.

Beside mutation of the p53 gene, other changes in the genome seen in breast cancers include amplification of certain dominant oncogenes, e.g. erbB, erbB2, erbB3, c-myc, H-ras, hst and int2 (reviewed in [17]). Amplification of DNA sequences in cells is suppressed by wild type p53, so that the mutational inactivation of p53 may also contribute to the progression of breast cancer by allowing DNA amplification.

p53: Human p53 is a 393 amino acid containing nuclear phosphoprotein. Both the germinal and the somatic mutations of p53 which are seen in cancers appear to be "loss of function" mutations. Several DNA tumor virus oncogenes, e.g. the E6 protein of the human papilloma virus or T Antigen of SV40 virus, specifically inactivate the p53 gene by protein-protein interactions. Likewise, a dominant cellular oncogene, mdm2, overexpressed in several sarcomas, exerts its tumorigenic function by interacting with and inactivating wild type p53 protein. Protein-protein interaction could be a common mode of inactivation of p53 in tumors which do not have somatic mutations in the p53 gene. Therefore, the overexpression of a cellular protein which interacts with p53 through its functionally significant domains may be as important for the pathogenesis of breast cancer as mutation of the p53 gene.

Presence of wild type p53 in a tumor is also important for responsiveness to chemotherapy and radiotherapy. The damage to DNA produced by these therapeutic agents leads to apoptosis of the cancer cells through a mechanism that requires wild type p53. This could be the reason behind the better prognosis of tumors with wild type p53 than those with mutated p53.

Overexpression of the wild type p53 protein arrests cell growth at a specific stage in the cell cycle, just before the onset of DNA replication at the G1-S boundary. Wild type p53 is also essential for DNA repair following radiation induced DNA damage, or for apoptosis of the cell if the DNA damage is extensive. The transforming mutants of p53 are defective in all these functions. Therefore of major concern in the field of breast cancer research is how the wild type p53 protein carries out these three diverse functions (inhibition of S phase, pause in DNA replication to repair damaged DNA, inhibition of DNA amplification). A simple hypothesis would be that p53 executes these diverse functions by directly inhibiting the DNA replication machinery. A clear understanding of the mechanism of action of p53 will allow (a) better diagnostic and prognostic tests for breast cancers, and (b) the design of a therapeutic strategy which restores p53 function in cancers with mutant p53.

Three mechanisms have been proposed by which p53 inhibits DNA replication. p53 has a sequence-specific DNA binding activity and an N terminal domain that behaves like a transcriptional activation domain. Indeed the p53 binding sites present upstream from a muscle creatinine kinase promoter acts as a transcriptional enhancer in co-transfection experiments with plasmids expressing wild-type p53. Therefore one mechanism by which p53 could act as a suppressor of S phase is by the transcriptional induction of genes that negatively regulate cell growth. However p53 can also bind to a basal transcriptional factor, the TATA box binding protein (TBP), through the N terminal transcription activation domain, and inhibit basal transcription from TATA box containing promoters . This has led to the second suggestion: p53 inhibits the expression of key S phase activators. p53 also interacts with the SV40 TAg through the middle half of the p53 protein (p53 was discovered because of this interaction). Through this physical interaction p53 inhibits the helicase activity of T antigen and inhibits the SV40 based *in vitro* DNA replication. Therefore the third possibility is that p53 directly interacts and inhibits a cellular origin binding protein replication initiator protein (as yet unidentified).

p53 physically interacts with and inhibits the function of the DNA replication factor RPA (described in the next paragraph) [18]. Thus p53 could directly interact with and regulate the DNA replication machinery. RPA binds to two separate domains of p53: an N-terminal transcriptional activation domain , and a C terminal domain that overlaps with the nuclear localization signal.

RPA: RPA (RF-A or human ssb; RPA stands for replication protein A) is a complex of three polypeptides of 70 kD, 34 kD and 13 kD, essential for SV40 DNA replication *in vitro* and excision repair in animal cells. The 70 kD subunit from human cells binds to single stranded DNA, and supports unwinding of the SV40 ori, but is

unable to support SV40 DNA replication *in vitro*, implying that the 34 and 13 kD subunits execute a function essential to DNA replication. Monoclonal antibodies against the 34 kD subunit inhibit DNA replication *in vitro*, again suggesting that the holocomplex RPA carries out functions essential to DNA replication through the 34 kD subunit. In *S. cerevisiae* the genes for the 70, 34 and 13 kD subunits are each essential for viability. The individual 70, 34 and 13 kD protein subunits are referred to as Rpa1, Rpa2 and Rpa3, respectively, and the genes are referred to as *Rpa1*, *Rpa2* and *Rpa3*.

Regulation of RPA by direct interaction with p53. As mentioned above, we have recently discovered that p53 physically interacts with and inhibits the DNA binding activity of RPA. This suggests a novel mechanism by which p53 inhibits DNA replication and executes the diverse activities which are lost in breast cancers (growth repression, pause in DNA replication for repair of damaged DNA and repression of DNA amplification).

Most of the transforming mutants of p53 fail to inhibit DNA replication *in vivo* and *in vitro* and fail to bind T antigen or to the specific p53 binding DNA element. However, the two transforming mutants of p53 we examined (R175H and R273H) could still interact with and inhibit RPA *in vitro*. Therefore the ability to interact with RPA is not the sole mechanism by which p53 inhibits cellular DNA replication, because mutant p53 which can still bind RPA fails to inhibit replication. We hypothesize that p53 has to be capable of binding RPA *and* DNA to effectively inhibit the DNA replication apparatus. The transforming mutants of p53 do not bind DNA and so fail to be near origins of replication where the inhibition of RPA has the most profound effect on DNA replication. In this hypothesis, the ability to interact with RPA is still important for the replication inhibitory property of p53, and the prediction is that point mutant forms of p53 which can bind to DNA, but do not bind RPA, will have lost the growth suppression function.

This brief description is intended to highlight two issues that are fundamental to this application. (1) Mutational inactivation of p53 is a common feature in the initiation and progression of breast cancers. The interaction of a known replication factor RPA with a tumor suppressor protein p53, is a novel finding that should be investigated in greater detail. The interaction is likely to have important consequences for the mechanism of growth suppression by p53 and may provide a novel therapeutic target (RPA) for breast cancers with mutated p53. (2) Though p53 is mutated in up to 50% of breast cancers, a significant fraction have wild type p53. In these cancers p53 could be inactivated by interaction with a mutant or over-expressed cellular protein that either masks the transcriptional activation domain of p53, or inhibits its nuclear localization domain. Therefore, RPA p70, which interacts with both these domains of p53, could inactivate p53 and play a direct role in breast cancer tumorigenesis.

BODY

SPECIFIC AIMS FOR YEAR 1

1. Define the part of p53 involved in binding RPA and mutate it to obtain mutant forms of p53 which do not bind RPA. (Task 1)
2. Analyze the effect of these mutations on the transcriptional property of p53. (Task 2a)
3. Define the part of Rpa1 that binds p53. (Task 3, first half)

METHODS

Plasmid constructions for making deletions of p53. Deletions of p53 were made by PCR with the appropriate primers such that a fragment of DNA encoding the relevant portion of p53 was made and cloned into pGTK between BamHI and SalI sites. The resulting plasmid expressed the deletion fragment of p53 fused in frame to GST. The protein was expressed in a standard strain of E. coli, and the fusion protein purified by affinity on glutathione agarose beads. The point mutations that changed W53 to S, and F53 to S were also made by PCR with appropriate mutagenic oligonucleotides. In this case the mutant form of p53 was also cloned into a mammalian expression vector, pcDNA3, which expressed the mutant p53 (without any N terminal fusion) upon transfection in SaOs2 cells.

Plasmid constructions for making deletions of Rpa1. The deletion derivatives of Rpa1 were made as follows. p1-616 was the original phRPA1 clone obtained from Dr. T. Kelly where the RPA1 cDNA is cloned into pKS- between EcoRI sites such that the RPA1 gene is downstream from the T7 promoter. The EcoRI fragment was re-cloned into pKS+ in the reverse orientation to obtain prevRPA1. phRPA1 was cut with ClaI (sites in the untranslated region downstream from RPA1 and in the polylinker) and ligated to obtain phRPA1ΔCla . phRPA1 was cut with HindIII or with XhoI and ligated to obtain p1-219 and p1-307, respectively. phRPA1ΔCla was cut with HindIII and ligated to obtain pΔ223-411. phRPA1 was cut with BclI and EcoRV (in the polylinker), the ends filled in and ligated to obtain p1-491. prevRPA1 was cut with XbaI or with SspI and ligated to obtain p1-370 and p1-521 respectively. prevRPA1 was cut with XhoI and ligated to obtain p349-616, and phRPA1ΔCla cut with PstI and ligated to obtain p278-616.

Site-directed mutagenesis of RPA1. The Stratagene PCR Site-directed Mutagenesis Kit was used to generate pm1-616. Primer RPA70 S3V4 (5'AAATTCGGTGTCGACCTTCTCAGAGCGGTA CAATCC3'), is complementary to human RPA1 sequence 1555-1590 with underlined nucleotides changed from wild type sequence. Primer 1591-1621 is the same as the corresponding sequence of human RPA1. Primer 1591-1621 was 5'-phosphorylated and 15 pmole of each primer used for PCR on 0.5 pmole phRPA1 template DNA using Taq polymerase and Taq extender. The PCR cycling parameters are as follows: Segment 1: 1 cycle of 94°C 4 min, 50°C 2 min, 72°C min; segment 2: 8 cycles of 94°C 1 min, 56°C 2 min, 72°C 1 min; segment 3: 1 cycle of 72°C 5 min. By keeping the number of cycles low the chances of unintentional PCR induced mutation is decreased. The PCR reaction is thus used to create a linear DNA fragment corresponding to the whole phRPA1 plasmid except the mutations incorporated in the primers. 1 μl of DpnI (which cuts methylated bacterial DNA) and cloned Pfu DNA polymerase were added at 37°C for 30 min to simultaneously select against the parental template DNA and to polish the ends of products respectively. After heat inactivation of the enzymes (72°C for 30 min) the PCR product was circularized by ligation and transformed into E. coli. The resulting plasmids were screened for the incorporation of

the SalI site designed in the mutagenic primer, and candidate plasmids sequenced to confirm the mutation and rule out adjoining secondary mutations.

In vitro transcription-translation and binding assays. Radio labeled protein was synthesized with 35S methionine and the Promega TnT Coupled *in vitro* transcription-translation kit. For measuring the binding of Rpa1 to p53, the translation mixes were diluted 1:10 with NETN containing 50 mM NaCl and 10 mg/ml Blotto. After incubation for 1 hr at 4°C with glutathione agarose beads coated with approximately 1 μg of GST (glutathione-S-transferase) or GST-p53, the beads were washed with 4x1 ml NETN (50 mM NaCl). Bound proteins were eluted by boiling the DNA-cellulose pellet in Laemmli's buffer, electrophoresed on 15% SDS-polyacrylamide gel and visualized by fluorography in 1M Sodium salicylate.

RESULTS

Sub-domains of p53 synergize to give strong RPA binding: GST fusion proteins containing various fragments of p53 were generated, bound to glutathione agarose beads and their ability to bind RPA examined by affinity chromatography (Fig. 1). We have shown earlier that two domains of p53, N2 (amino acids 2-121) and 5C (amino acids 289-393), could independently bind RPA. Amino acids 2-71 of p53 (a domain) had equivalent RPA binding activity as 2-121 (data not shown). However amino acids 2-45 or 46-71 (sub-domains) showed much reduced RPA binding activity. Similarly at the C terminal end, while 289-356 had significant RPA binding, 289-330 or 331-356 did not have significant RPA binding. Ten times as much of each GST-sub-domain protein (e.g. GST 2-45 or GST 46-71) were compared to GST-domain protein (GST 2-71) in their ability to bind RPA. The binding activity of each sub-domain was less than one-tenth that of the corresponding domains. Thus the better binding of RPA by a domain (e.g. 2-71) is probably not a simple summation of RPA binding by each of the sub-domains (e.g. 2-45 and 46-71). Similar results were obtained with 289-356 (data not shown). It is unlikely that in two separate instances the absence of RPA binding by the sub-domains is due to the RPA binding site spanning the site of division. The alternative explanation is that weak RPA binding sites in each of the sub-domains synergize to produce the strong binding activity of the corresponding domain.

Further mapping of the RPA binding site of p53 (see below) suggests that amino acids 48, 49, 53 and 54 are important for the interaction. Therefore, the failure of 46-71 to bind RPA may be because the fusion with GST at amino acid 46 inadvertently changes the structure of the RPA binding site or even truncates it.

Aromatic amino acids in a sub-domain of p53 are important for RPA binding: The transcriptional trans-activator VP16 has been shown to interact with RPA, and a phenylalanine to proline mutation in VP16 shown to diminish RPA binding. Reasoning that a similar mechanism of interaction occurred between RPA and p53, point-mutations were made in p53 which changed two adjoining aromatic amino acids, tryptophan and phenylalanine (residues 53-54) in one of the sub-domains of N2 to serines (W53S-F54S). This GST fusion protein did not bind RPA (data not shown). Several other point mutations have also been made in the N terminal part of p53 in the laboratory of Dr. A. Levine, and a representative collection of these and W53S-F54S were engineered into GST-p53 fusion proteins and their RPA binding activity determined (Fig. 2). The results demonstrate that the aromatic residues W53 and F54 are important for RPA binding. Mutations in amino acids 48-49 (D48H-D49H) also decreased RPA binding, suggesting that negatively charged amino acids near the hydrophobic residues at 53-54 were important for RPA binding. The mutations which changed amino acids 22-23 of p53 (L22Q-W23S) affect its ability to activate transcription [19], but did not affect its ability to bind RPA. Thus although in the herpesvirus transcriptional activator VP16 the same amino acid (F442) is important for both interaction with RPA and activation of

transcription, this is not the case with p53. Therefore it seemed likely that we can separate the trans-activation and RPA binding functions of p53 with appropriate point-mutations.

RPA from crude cell extracts does not bind to the 5C domain of p53: The W53S-F54S mutation in p53 produced a significant decrease in the binding of RPA from crude cell extracts (S100 extracts). This result was unexpected because the C terminal 5C domain of p53 had also been shown to interact with purified RPA. One explanation could be that 5C is unable to bind RPA from S100 extracts. When tested directly, we found that while N2 could bind RPA from both purified fractions and from cell extracts, 5C could only bind RPA from the former (Fig. 3). Although we do not know why the 5C part of p53 does not bind RPA from crude cell extracts, the above observation explains how we obtained a mutant form of p53 which loses the ability to bind RPA from cell extracts by making mutations only in the N2 domain of p53. This approach has the added advantage of leaving intact the dimerization and nuclear localization functions in the 5C domain which are essential for growth suppression.

Specific interactions of the C terminal portion of p53 with other products in the S100 extract could be responsible for the failure of p53, 5C, to interact with RPA in the crude extract. However, pre-incubation of GST-5C with S100 extract, washing off unbound proteins and then adding pure RPA did not block the binding of RPA to 5C. In the reverse experiment, pure RPA was added to S100 extract and then asked to bind to 5C. Again the association of pure RPA to 5C was not affected by the S100 extract. Hence at the moment we do not understand why the RPA in the S100 extract fails to bind to 5C. The best hypothesis is that the RPA present in the S100 extract is in a complex with some cellular protein which prevents it from interacting with 5C.

Transcription activation by p53 mutants: To test the transcription activation properties of these p53 molecules, a transient transfection assay was done (Fig. 4). Plasmids expressing p53 and various mutant derivatives were co-transfected into SaOs2 cells with a reporter plasmid, 6FSVCAT, which has six consensus p53 binding sites cloned upstream from a CAT gene. Only the L22Q-W23S mutation of p53 significantly affected transcription activation by p53, although it still retained five-fold activation over vector control. D48H-D49H, W53S-F54S and D61H-E62K mutant forms of p53 retained 50-100% of transcriptional activity compared to wild-type p53. In D48H-D49H and W53S-F54S we had versions of p53 with near wild-type trans-activation but significantly diminished RPA binding activities.

Region of Rpa1 required for binding p53. We have reported that RPA bound to p53 fails to bind single-stranded DNA [18]. One explanation could be that the overlapping regions of Rpa1 are required to bind the two ligands, so that the ligands are mutually exclusive. To determine if this was the case, we used the deletion derivatives of Rpa1 to map the region required to bind to p53. Rpa1 and deletion derivatives were synthesized in vitro and bound to glutathione agarose beads coated with either glutathione S transferase (GST) or GSTp53 (Summarized in Fig. 5). In contrast to the binding region of ssDNA or Rpa2, the binding region of p53 was difficult to map from the deletions of Rpa1. A small C terminal deletion increased binding to p53 (1-616 v. 1-521) suggesting the presence of an inhibitory domain at the extreme C terminus. Due to the weak binding of 1-370 the p53 binding region may already be affected by this deletion. The inability of 278-616 to bind p53 also suggests that residues 1-278 is essential for p53 binding.

Overall, the deletion derivatives that contained the 1-521 region of Rpa1 could bind p53 The existence of inhibitory (521-616) domains which influence binding to GST-p53 was unexpected.

The putative zinc finger of Rpa1 is dispensable for binding single-stranded DNA, p53 or Rpa2. A putative C4-type zinc finger motif was noted at position 481-503 of human Rpa1, which is evolutionarily conserved in yeast Rpa1. Since 1-491 could weakly bind p53, the zinc finger is also dispensable for binding p53. To confirm whether the zinc finger was important for binding p53, point-mutations were made in the Rpa1 cDNA which changed the 2 C terminal cysteines of the putative zinc finger to serines (m1-616). This point-mutated form of Rpa1 was synthesized in vitro and its ability to bind p53 measured by pull-down with GST-p53 beads. The mutated form of Rpa1 associated with p53 as effectively as wild-type Rpa1 (data not shown). Therefore, the putative zinc finger is not required for binding p53.

FIGURE LEGENDS

Fig. 1 Binding of RPA by GST fusion proteins containing the indicated portions of p53 (e.g. amino acids 289-356 etc.) GST: glutathione S transferase without any p53 fusion. 0.1 input: one-tenth of the input RPA mixed with glutathione beads containing equal quantities of the indicated GST proteins. After incubation and washing the RPA bound on the beads is visualized as described in Ref. 18. On the right hand side we show that even when one-tenth as much 2-71 (third lane from right) is used as 2-45 or 46-71, the association of RPA is stronger with 2-71.

Fig. 2 Binding of RPA from purified RPA preparation (RPA) or from crude cell extract (S100) by GST fused to amino acids 2-117 of p53 (N2) or amino acids 289-393 of p53 (5C). p70 and p34 are Rpa1 and Rpa2 respectively. The rest is as in Fig. 1.

Fig. 3 Binding of RPA by GST fusion proteins containing mutant alleles of p53 with the indicated amino acids mutated by site-directed mutagenesis. wt p53: wild type p53. 61-62: D61H-E62K where the aspartic acid (D) at position 61 is changed to histidine (H) and so on, 53-54: W53S-F54S, 48-49: D48H-D49H, 22-23: L22Q-W23S, 14-19: L14Q-F19S. The rest is as in Fig. 1.

Fig. 4 Transcription transactivation by various alleles of p53 assayed by transient transfection of plasmids expressing p53 with FSVCAT, a plasmid containing the CAT gene under control of a p53 responsive promoter, into p53 null SaOs2 and H1299 cells. cDNA3: vector alone, wt: cDNA3 containing wild-type p53. The rest as in Fig. 3.

Fig. 5 Summary of binding of Rpa1 with Rpa2, p53 and single-stranded DNA cellulose (the last only at 0.5M NaCl). Binding of Rpa1 and derivatives to GST-p53 was measured as described in the text. The Rpa1 and deletion derivatives are represented by rectangles of appropriate length and the amino acids at the limits of the deletions indicated. Binding of the same derivatives to single-stranded DNA and to Rpa2 are not the subject of this grant, but are indicated for comparison with the p53 binding region. +: binding the same as wild-type Rpa1 (1-616), with ++ indicating binding better than wild-type, and +/- indicating binding less than wild-type.

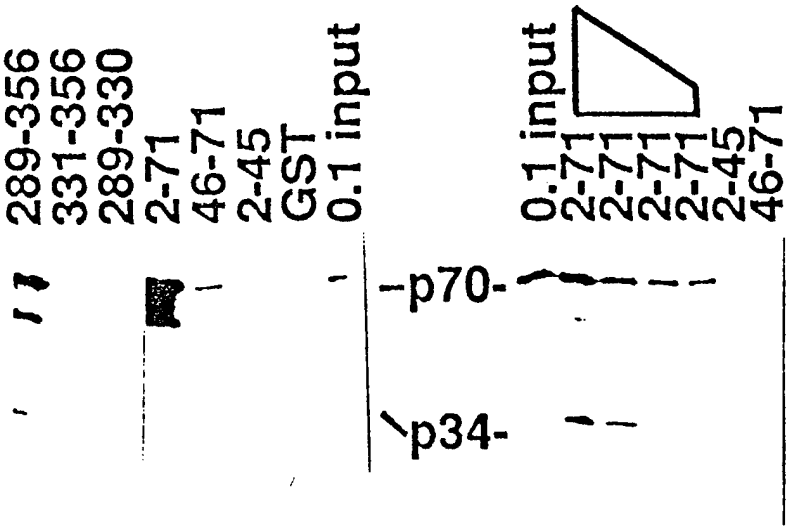
289-356
331-356
289-330
2-71
46-71
2-45
GST
0.1 input
0.1 input
2-71
2-71
2-71
2-71
2-45
46-71
p70-
p34-

Figure 1

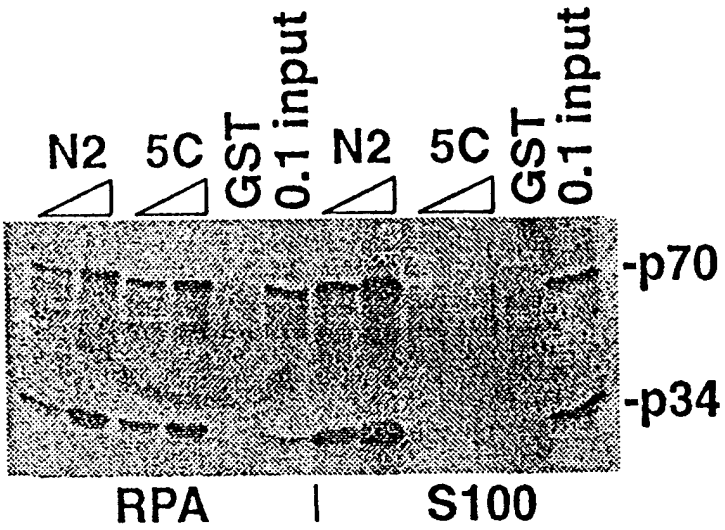

Figure 2

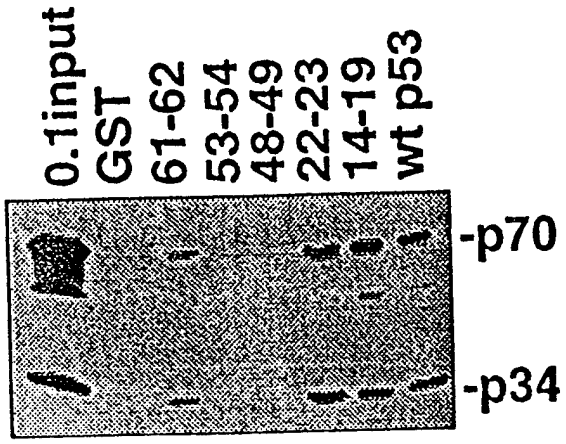

Figure 3

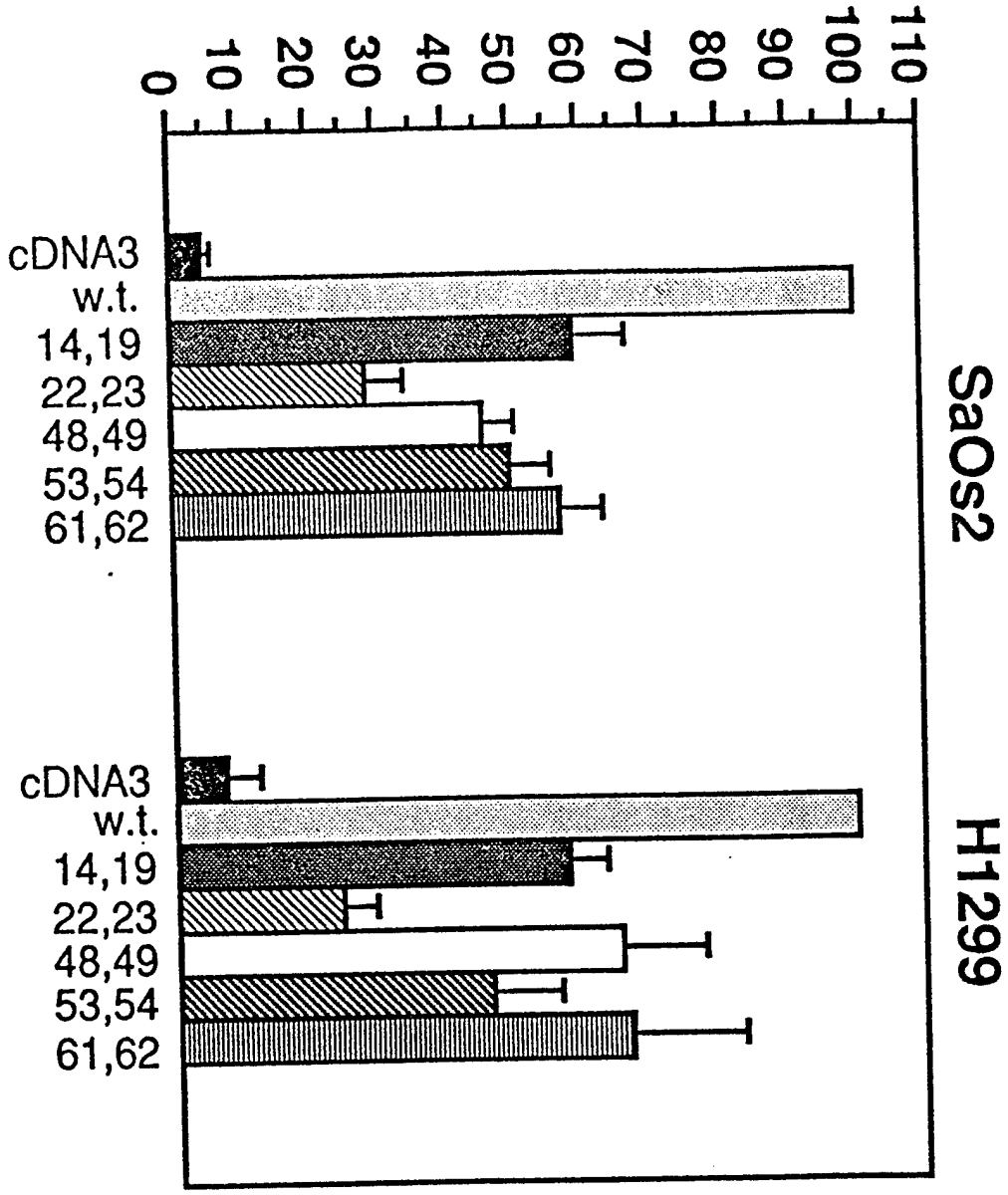
% chloramphenicol acetylated (%w.t.)
0
10
20
30
40
50
60
70
80
90
100
110
cDNA3
w.t.
14,19
22,23
48,49
53,54
61,62
SaOs2
cDNA3
w.t.
14,19
22,23
48,49
53,54
61,62
H1299

Figure 4

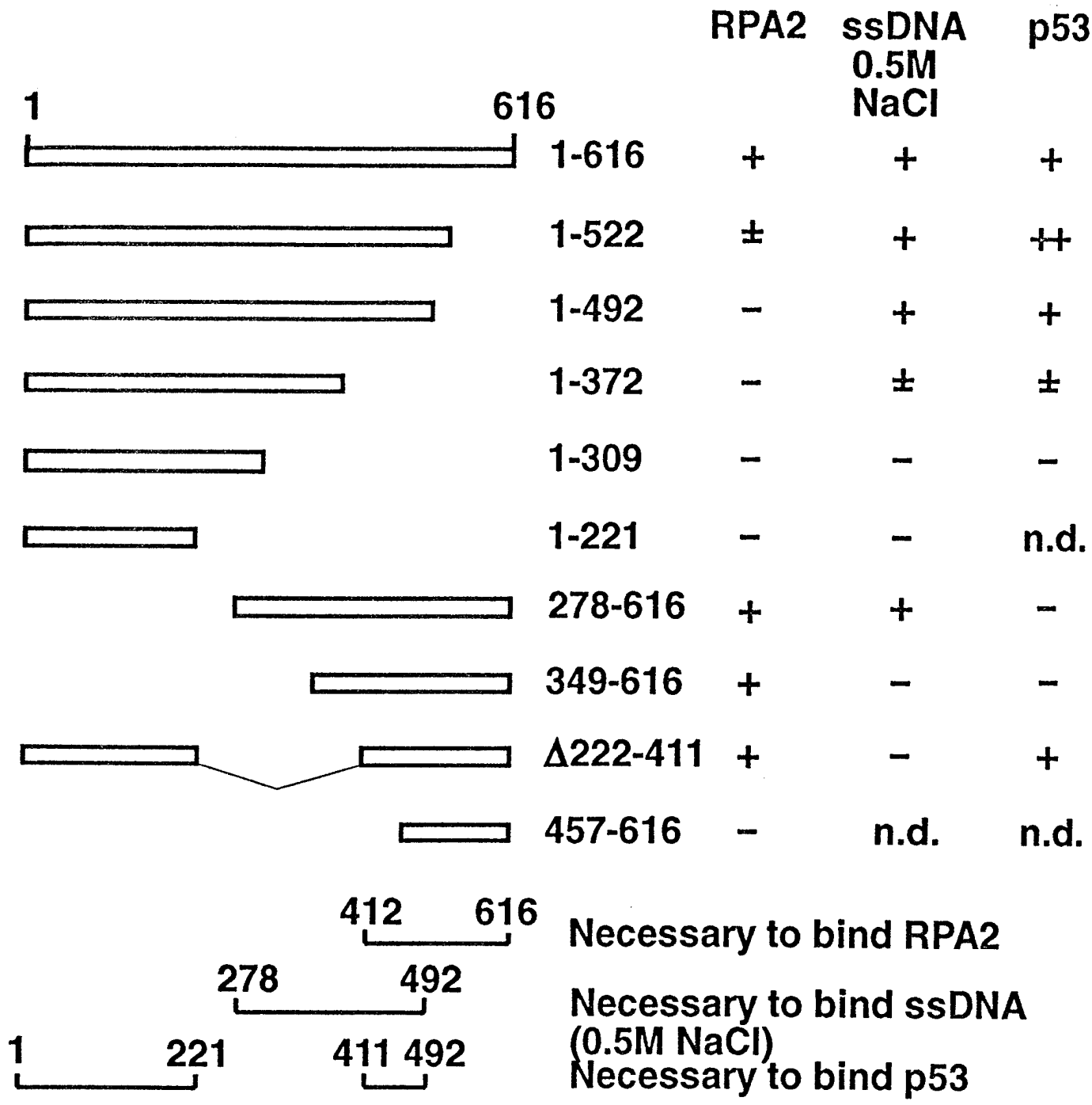
RPA2
ssDNA 0.5M NaCl
p53
1
616
1-616 + + +
1-522 ± + ++
1-492 – + +
1-372 – ± ±
1-309 – – –
1-221 – – n.d.
278-616 + + –
349-616 + – –
Δ222-411 + – +
457-616 – n.d. n.d.
412 616
278 492
1 221
411 492
Necessary to bind RPA2
Necessary to bind ssDNA (0.5M NaCl)
Necessary to bind p53

Figure 5

CONCLUSIONS

Relatively short peptides with aromatic amino acids surrounded by negatively charged residues produce RPA binding activity when repeated several times in a protein. The degenerate nature of this RPA binding signal probably accounts for the large number of proteins reported to bind RPA. Since similar bulky hydrophobic residues in the context of negatively charge amino acids are also essential for transcriptional trans-activation, this observation may explain why several transcriptional activators are able to bind RPA. Potentially, this could be a common feature of several protein-protein interactions, although at present it is restricted to interactions between transcription/replication regulatory proteins, and proteins of the basal transcription or replication apparatus. The recently reported interaction between the DNA repair protein XP-G and RPA also uses a negatively charged part of XP-G, and it is possible that the same mode of interaction is involved [20].

Bulky hydrophobic residues in the N terminal region of p53 have already been implicated in transcriptional trans-activation and in interactions with the TATA binding protein and viral and cellular oncogenes E1b and mdm2 respectively [19]. Although our results suggest that at least for RPA binding the interaction depends on relatively degenerate sequences, there must be some specificity to the modules that interact with transcriptional factors versus the ones that interact with RPA because we were able to create point mutation which affected RPA binding but not trans-activation and vice-versa. The exact source of this specificity will become more clear when the interaction domains of the other partners (RPA, transcriptional co-activator, E1b or mdm2) in these interactions are defined.

Peptides of varying lengths could be multimerized to produce RPA binding. Therefore, there do not appear to be strict structural constraints on the interactor modules, because each of the multimers have different distances between the aromatic residues, between the acidic residues and between the aromatic and acidic residues. We think that the interactor modules are unstructured, and may be induced into a more defined structure when the other partner is bound. This "induced fit" hypothesis also leaves room for specificity of interaction depending on the structure of the other partner in the interaction. One could speculate that the minimal requirements of the other partner would be to have a distribution of bulky hydrophobic residues surrounded by positively charged residues, so that hydrophobic and electrostatic interactions would stabilize the interaction.

L22Q-W23S showed decreased transcription activation but wild-type RPA binding indicating that transcriptional trans-activation function is not required for RPA binding. On the other hand, D48H-D49H and W53S-F54S showed wild-type transcription activation and lost RPA binding, confirming that the RPA binding site of p53 is different from its transcription trans-activating site. It will be interesting to determine how these mutation of p53 affect (a) its ability to repress transcription, (b) ability to suppress growth of cells, and (c) ability to induce apoptosis.

Scrutiny of the Rpa1 deletions shows that association with p53 did not correlate with binding to single-stranded DNA or to Rpa2. There was a derivative which bound p53 well but not single-stranded DNA (349-616) and others which bound both well (1-521). Likewise there was an Rpa1 derivative which bound Rpa2 well but not p53 (Δ223-411), two that did the reverse (1-521 and 1-491) and another that bound both well (349-616). This confirms our previous observation that the Rpa1-p53 interaction did not require Rpa2 or single-stranded DNA [18]. The failure of 278-616 to bind p53 puts the N terminal limit of the p53 binding domain N terminal to amino acid 278. The C terminal limit of the minimal p53 binding domain is probably between residues 491 and 521. We have also defined the minimal domains of Rpa1 that are required to bind Rpa2 and single-stranded DNA, and the results suggest that the p53 binding site significantly overlaps with the sites for binding DNA, but not with the site for binding Rpa2. This

explains why p53 bound RPA remains as a holocomplex (Rpa1+Rpa2+Rpa3) but fails to bind single-stranded DNA.

The ability to divide the Rpa subunits into sub-domains required for essential activities (holocomplex formation and DNA binding) opens the way toward screening for point mutations in Rpa1 which can still form the holocomplex, and bind DNA, but cannot bind p53 or other transcriptional activators. Should such a mutant form of Rpa1 be obtained, we shall be able to test the importance of the p53-Rpa1 interaction on RPA function in replication, repair and recombination or the regulation of these processes by the reported interactions with p53, transcriptional activators and DNA repair proteins.

Overall the project is progressing on schedule and the reagents generated will help us answer the questions we set out to address in the original grant proposal. Our lab has made additional progress on other aspects of p53 function and RPA. Specifically, we have demonstrated that a transcriptional target of p53, the p21 protein interacts directly with another DNA replication factor, PCNA, and in so doing inhibits DNA replication [21]. This could be a novel mechanism by which p53 keeps the DNA replication and repair apparatus in control in normal mammary epithelial cells. We have also discovered a homolog of the middle subunit of RPA, tentatively named Rpa4 [22]. We will at a future date test whether this novel form of RPA composed of Rpa1+Rpa4+Rpa3 has similar p53 binding properties as the authentic RPA (Rpa1+Rpa2+Rpa3).

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